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1 **Circadian oscillations of cytosolic free calcium regulate the Arabidopsis**

2 **circadian clock**

3

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29

30 **Abstract**

31 In the last decade, the view of circadian oscillators has expanded from transcriptional
32 feedback to incorporate post-transcriptional, post-translational, metabolic processes
33 and ionic signalling. In plants and animals, there are circadian oscillations in the
34 concentration of cytosolic-free Ca^{2+} ($[\text{Ca}^{2+}]_{\text{cyt}}$), though their purpose has not been fully
35 characterised. We investigated whether circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ regulate the
36 circadian oscillator of *Arabidopsis thaliana*. We report that in *Arabidopsis*, $[\text{Ca}^{2+}]_{\text{cyt}}$
37 circadian oscillations can regulate circadian clock function through the Ca^{2+} -dependent
38 action of CALMODULIN-LIKE24 (CML24). Genetic analyses demonstrate a linkage
39 between *CML24* and the circadian oscillator, through pathways involving the circadian
40 oscillator gene *TIMING OF CAB2 EXPRESSION1* (*TOC1*).

41

42 Circadian oscillators confer competitive advantage by modulating physiology and
43 development^{1, 2}. In Eukaryotes, circadian oscillators are comprised of feedback loops of
44 transcriptional regulators, however, the oscillator genes differ between the Kingdoms².
45 ³. In *Arabidopsis thaliana*, a morning loop is formed of *CIRCADIAN CLOCK*
46 *ASSOCIATED1* (*CCA1*)⁴ and *LATE ELONGATED HYPOCOTYL* (*LHY*)⁵, *PSEUDO*
47 *RESPONSE REGULATOR 7* (*PRR7*) and *PRR9*⁶. The evening feedback loop involves
48 *TIMING OF CAB2 EXPRESSION1* (*TOC1*)⁷ and *GIGANTEA* (*GI*)⁸. These loops are
49 connected through *CCA1/LHY* mediated repression of *TOC1* expression⁹ and *TOC1*-
50 mediated repression of *CCA1* involving *CCA1 HIKING EXPEDITION* (*CHE*)¹⁰.

51

52 Circadian oscillators also incorporate post-transcriptional, post-translational and
53 metabolic processes¹¹⁻¹⁶. In *Arabidopsis*, these include the F-box protein ZEITLUPE
54 (ZTL)¹⁷ regulated blue-light dependent degradation of *TOC1*^{15, 18, 19} and photosynthetic
55 sugars affect entrainment¹¹. Several studies have revealed the importance of ionic
56 signalling for circadian timekeeping²⁰⁻²³. In *Drosophila*, circadian oscillations of

intracellular Ca^{2+} ($[\text{Ca}^{2+}]$) regulate cellular oscillations *in vivo*²³ and temperature-induced increases in cytosolic $[\text{Ca}^{2+}]$ are involved in entrainment²¹.

In plants, like in the mammalian suprachiasmatic nucleus, there are circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ ²⁴. They are driven by cyclic adenosine diphosphate ribose-mediated Ca^{2+} -release from internal stores^{16, 25-28} to encode information about photoperiod, timing and light intensity^{29, 30}. However, the functions regulated by circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ have not been identified. Therefore, it has been conjecture whether circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ represent an input to the oscillator or part of the timing mechanism, in addition to being an output²⁴.

We show that circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ affect the abundance of *CHE* and affect circadian period through a Ca^{2+} -dependent regulatory protein of the plant specific CALMODULIN-LIKE (CML) family. We conclude that CML24 is part of the Arabidopsis circadian system, acting through a Ca^{2+} -dependent pathway to regulate *TOC1*.

Results

Circadian oscillator gene expression can be altered by $[\text{Ca}^{2+}]_{\text{cyt}}$ signals

We identified potential targets for $[\text{Ca}^{2+}]_{\text{cyt}}$, by examining by microarray the response of circadian oscillator transcripts to a single 24 h artificial oscillation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in plants in which circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ were abolished, and later artificially induced (Fig. 1a and Supplementary Fig. 1). $[\text{Ca}^{2+}]$ signals did not restore high amplitude oscillations of clock transcripts like those in light and dark cycles³¹. *CCA1 HIKING EXPEDITION* (*CHE*) was the only clock transcript whose abundance correlated with the $[\text{Ca}^{2+}]_{\text{cyt}}$ signal, having a dynamic opposite to the imposed $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillation (maximum repression 5.2 fold at 12 h, 4.5 fold at 16 h and 3.1 fold at 8 h) (Fig.1b). The *CCA1* dynamic was modestly altered (1.9 fold activation at 20 h and 24 h) (Fig. 1b). This later increase in *CCA1* transcript abundance might have been due to the earlier

85 large repression of *CHE*. Artificial $[Ca^{2+}]$ oscillation had smaller effects on other
 86 circadian oscillator transcript abundance, the largest being a 2 fold reduction of *PRR3*
 87 at 16 h and a 1.9 fold increase of *LHY* at 20 h (Fig. 1b).
 88 To test further the effects of $[Ca^{2+}]_{cyt}$ on *CHE* transcript abundance, we screened a
 89 number of $[Ca^{2+}]_{cyt}$ agonists to identify those that had profound and persistent effects
 90 on $[Ca^{2+}]_{cyt}$. N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7)
 91 in combination with $CaCl_2$ evoked sustained increases in $[Ca^{2+}]_{cyt}$ ³². This Ca^{2+} agonist
 92 caused a transient and large rise of $[Ca^{2+}]_{cyt}$ peaking at 848.5 ± 156.0 nM, followed by a
 93 sustained elevation for at least 2 h (Supplementary Fig. 1). Induction of large,
 94 sustained $[Ca^{2+}]_{cyt}$ increases at ZT36 and ZT48 by 2 h treatment with W7 and $CaCl_2$,
 95 confirmed that the major transcriptional response of the circadian oscillator was a
 96 repression of *CHE* abundance (Fig. 1c). Similar to the experiment with a single artificial
 97 24 h $[Ca^{2+}]_{cyt}$ oscillation, the abundance of morning transcripts *CCA1* and *LHY* did not
 98 alter immediately after the $[Ca^{2+}]_{cyt}$ increase (Fig. 1c). This increase in $[Ca^{2+}]_{cyt}$
 99 significantly altered the abundance of transcripts expressed later in the day, being an
 100 activator of *PRR9* and a repressor of *PRR7*, *PRR3* and *CHE* (fold changes: *PRR9* 3.2
 101 (ZT48), *PRR7* 2.0 (ZT48), *PRR3* 1.7 (ZT36) and 2.1 (ZT48), *CHE* 3.4 (ZT36)) (Fig. 1c).
 102 Finally, the transcript levels of the evening genes were not affected (Fig. 1c). These
 103 Ca^{2+} -induced changes in *CHE* transcript did not alter the free running period of the
 104 oscillator, because treating plants with the W7 solution at ZT0 and ZT12 did not alter
 105 the period of *CCA1:LUC* (ZT0 Control 24.4 ± 0.5 h, W7 solution 24.2 ± 0.2 h; ZT12
 106 Control 24.4 ± 0.2 h, W7 solution 24.4 ± 0.5 h; $p > 0.05$). Thus, manipulation of $[Ca^{2+}]_{cyt}$
 107 demonstrated that both 24 h $[Ca^{2+}]_{cyt}$ oscillations and shorter-term $[Ca^{2+}]_{cyt}$ signals can
 108 regulate *CHE* transcript abundance (Fig. 1).
 109
 110 **A reverse genetic screen identified *Calmodulin-Like 24 (CML24)* as a regulator of**
 111 **circadian period**

112 Because transient increases in $[Ca^{2+}]_{cyt}$ affected circadian oscillator gene abundance
 113 but not period, we wished to determine whether Ca^{2+} signalling has a role in regulating
 114 the clock *in planta*. We performed a screen of 75 well characterised Ca^{2+} signalling
 115 mutants of transporters, transducers and sensors. Five lines had significantly increased
 116 circadian period of leaf movement compared to wildtype (Fig. 2 and Supplementary
 117 Table 2); *vitamin C (vtc) 1-1*³³ (Col-0 23.8 ± 0.1 h, *vtc1-1* 24.7 ± 0.2 h, p=0.001),
 118 *calmodulin-like (cml) 24-1* and the double mutant *cml23-2 cml24-1* (Col-0 24.1 ± 0.1 h,
 119 *cml24-1* 24.6 ± 0.1 h, *cml23-2 cml24-1* 25.0 ± 0.1 h, p<0.001 for both) and also *cml24-*
 120 *4* and the double mutant *cml23-2 cml24-4*^{34, 35} (Col-0 23.9 ± 0.1 h, *cml24-4* 25.1 ± 0.1
 121 h, *cml23-2 cml24-4* 25.6 ± 0.1 h, p<0.001 for both). *cml23-2* only had a phenotype
 122 when in combination with alleles of CML24 (Fig. 2), suggesting that CML24 and CML23
 123 could be redundant in the regulation of the clock. None of the overexpressing (*CML24-*
 124 *OX1* and *CML24-OX2*) or underexpressing lines (*CML24-U1* and *CML24-U1*) affected
 125 circadian period (Supplementary Table 2). As *CML24* (previously called *TCH2*)^{36, 37}
 126 encodes a CALMODULIN-LIKE Ca^{2+} -sensor^{34, 35} and two different alleles, *cml24-1* and
 127 *cml24-4* had a significantly longer period than Col-0 for leaf movements (Fig. 2), we
 128 decided to further characterize whether CML24 is involved in the regulation of the
 129 circadian oscillator. The long circadian period of *cml23-2 cml24-4* double mutants was
 130 confirmed by measuring promoter activity of *CCA1* fused to luciferase (*CCA1:LUC*) and
 131 circadian oscillations of $[Ca^{2+}]_{cyt}$ (Fig. 3a-3d) (Col-0 23.7 ± 0.1 h, *cml23-2 cml24-4* 26.1
 132 ± 0.4 h for *CCA1:LUC*, p<0.001; Col-0 23.7 ± 0.2 h, *cml23-2 cml24-4* 25.1 ± 0.1 h for
 133 *35S:AEQ*, p<0.001). To investigate the effect of *CML24* on the central oscillator in
 134 more detail, we analyzed *CCA1*, *PRR7* and *TOC1* transcript abundance in Col-0 and
 135 *cml23-2 cml24-4*. In the third day in LL, there was a substantial delay of 4 h in the
 136 phase of *CCA1*, *TOC1* and *PRR7* transcript abundance in the mutant plants (Fig. 3e),
 137 consistent with the lengthening of circadian period by ~1.5 h described in Fig. 3a-d.
 138 The transcript levels of the clock components were unaffected.

139

CML24 regulates circadian period in a $[Ca^{2+}]_{cyt}$ -dependent manner

Because circadian rhythms persist in conditions where the circadian oscillations of $[Ca^{2+}]_{cyt}$ are abolished, such as nicotinamide¹⁶, sucrose²⁴ or monochromatic red light²⁹, circadian rhythms of $[Ca^{2+}]_{cyt}$ are not necessary for a rhythmic oscillator. However, we could test for necessity for oscillations in $[Ca^{2+}]_{cyt}$ in the correct regulation of circadian period by determining whether the action of CML24 in the circadian clock depends upon Ca^{2+} . If the effects of the CMLs are independent of Ca^{2+} , then the effects of mutation on circadian period should be additive to treatments that abolish circadian $[Ca^{2+}]_{cyt}$ oscillations. Whereas, if the action of the CMLs is dependent on Ca^{2+} , then mutation should have no further effect in the presence of Ca^{2+} antagonists. 50 mM nicotinamide increased the circadian period of leaf movement¹⁶ but in its presence, *cml23-2 cml24-4* was indistinguishable from the wild type (Col-0 27.7 ± 0.2 h, *cml23-2 cml24-4* 28.1 ± 0.2 h, $p=0.490$) (Fig. 4a). Sucrose abolishes $[Ca^{2+}]_{cyt}$ oscillations²⁴, however under high and low light conditions it has different effects on circadian period¹¹. There was no significant difference between *cml23-2 cml24-4* and wild type on 3% sucrose (high light Col-0 24.7 ± 0.1 h, *cml23-2 cml24-4* 25.0 ± 0.1 h, $p = 0.051$; low light Col-0 26.4 ± 0.2 h, *cml23-2 cml24-4* 26.9 ± 0.2 h, $p=0.061$) (Figs. 4b and 4c). Lastly, in monochromatic red light, the circadian period was not statistically different in the *cml23-2 cml24-4* mutant compared to Col-0 (Col-0 26.3 ± 0.4 h, *cml23-2 cml24-4* 26.1 ± 0.2 h $p=0.410$) (Fig. 4d). The lack of an effect of *cml23-2 cml24-4* in conditions that abolish circadian oscillations of $[Ca^{2+}]_{cyt}$ is consistent for loss-of-function mutations in putative Ca^{2+} -sensor proteins and CML24 acting downstream of $[Ca^{2+}]_{cyt}$.

Additionally, we found that *CML23* and *CML24* transcript abundance (Fig. 4e) was increased in response to the artificially imposed $[Ca^{2+}]_{cyt}$ oscillation (Fig. 1a), with their peaks in phase with the imposed $[Ca^{2+}]_{cyt}$ rhythm, suggesting $[Ca^{2+}]_{cyt}$ positively regulates *CML* abundance. This could be explained by the presence in their promoters of the Ca^{2+} -Responsive cis element CAM box (ACGCGT)³².

168 CML24 not only senses Ca^{2+} , it also regulates NO^{35} [35] as shown by high levels of NO
169 in *cml24* mutants³⁵. We therefore, tested whether the high NO in the mutants could be
170 the cause of the long period by two experiments. We investigated the effect of NO on
171 circadian regulation of $[\text{Ca}^{2+}]_{\text{cyt}}$ and *CHLOROPHYLL A/B BINDING*
172 *PROTEIN2:LUC* (*CAB2:LUC*) in wild type plants and found no evidence for a role for
173 NO, because the NO donor, SNAP, and the scavenger, cPTIO, were without effect on
174 circadian rhythms (Supplementary Fig. 2). The high NO levels found in *cml23-2 cml24-*
175 *4*, were not responsible for the long period phenotype, because the mutant long period
176 phenotype persisted in the presence of cPTIO (Fig. 4f)³⁸.

177 We conclude that the effect of the *cml23-2 cml24-4* mutations on the circadian clock
178 requires $[\text{Ca}^{2+}]_{\text{cyt}}$ and is independent of the effects of CML24 on NO generation.

179 **CML24 regulates circadian period by a pathway involving *TOC1* and possibly**
180 ***CHE***

181 To investigate how CML24 affects circadian period, we tested whether it is involved in
182 the $[\text{Ca}^{2+}]_{\text{cyt}}$ -mediated transcriptional regulation of circadian clock genes. The clock
183 transcripts regulated by $[\text{Ca}^{2+}]_{\text{cyt}}$ in Col-0 (Fig. 1c) were also regulated by $[\text{Ca}^{2+}]_{\text{cyt}}$ in
184 *cml23-2 cml24-4* (Supplementary Fig. 3), suggesting that CML24 is not involved in the
185 transcriptional regulation by $[\text{Ca}^{2+}]_{\text{cyt}}$.

186

187 We then investigated the genetic linkage between CML24 and components of the
188 oscillator. We studied epistatic interactions in the control of circadian rhythms of leaf
189 movement between mutations in *CML23/24* and *CCA1*, *LHY*, *TOC1*, *ZTL*, *ELF3*, *ELF4*
190 and *LUX*. Double *CML23/24* mutants were used for epistasis due to having a larger
191 measurable effect on period compared to the single CML24 mutants. We identified
192 epistasis between mutations in *CML23/CML24* and *TOC1* in the regulation of the
193 period of leaf movement (Fig. 5a, Supplementary Fig. 4 and Supplementary Table 3).
194 *toc1-2* single mutant had a short period (*C24* 24.3 ± 0.1 h, *toc1-2* 21.4 ± 0.3 h, Mann-

Whitney Rank Sum Test $p < 0.001$; Fig. 5a)²⁹. In the triple mutant *toc1-2 cml23-2 cml24-4*, the long period arising from mutations in *CML23/CML24* was absent, having a period that was indistinguishable from the single mutant *toc1-2*, and significantly shorter than *cml23-2 cml24-4* mutant (One-way ANOVA $p = 1$ and $p < 0.001$, respectively) (Fig. 5a, Supplementary Fig. 4 and Supplementary Table 3). This indicates that *toc1-2* is epistatic to *cml23-2 cml24-4*. We did not find epistatic interactions between mutations in *CML23/CML24* and *CCA1*, *LHY* or *ZTL*. *cml23-2 cml24-4* increased period in the *cca1-11*, *lhy-21* and *ztl-3* backgrounds, resulting in additive phenotypes (Fig. 5b-5d and Supplementary Table 3). Analysis of genetic interactions between *CML23/CML24* and *ELF3*, *ELF4* and *LUX* is complicated by the arrhythmic phenotypes caused by loss-of-function of these evening complex genes. Triple mutants of *cml23-2 cml24-4* and members of the evening complex were therefore all arrhythmic in LL (Supplementary Fig. 4). Crossing the wild type genetic backgrounds used in this study was without effect (Supplementary Table 3).

TOC1-mediated repression of *CCA1* involves *CHE*¹⁰. Therefore, the regulation of *CHE* by $[Ca^{2+}]_{cyt}$ and interaction between mutations in *CML23/CML24* and *TOC1*, prompted investigation of whether *CHE* is also part of the genetic pathway by which *CML24* regulates circadian period. We identified epistatic interaction between mutations in *CML23/CML24* and *CHE* in the regulation of circadian leaf movements. As previously reported for *CCA1:LUC⁺* rhythms, the circadian period of leaf movement in *che-1* and *che-2* single mutants was indistinguishable from the background (two-tailed Student's t-test $p = 0.461$ and $p = 0.681$, respectively; Fig. 6a and 6b)¹⁰. In the triple mutant *che-2 cml23-2 cml24-4*, the long period arising from mutations in *CML23/CML24* was absent, being indistinguishable from the single mutant *che-2* and significantly shorter than the *cml23-2 cml24-4* mutant (One-way ANOVA $p > 0.05$ and $p < 0.05$, respectively) (Fig. 6a, Supplementary Fig. 4 and Supplementary Table 3). However, we found no evidence that *che-1* was epistatic to *cml23-2 cml24-4*. *che-1 cml23-2 cml24-4* triple mutants had

223 a significantly longer period relative to *che-1* and similar to *cml23-2 cml24-4* (One-way
224 ANOVA, $p < 0.05$ and $p > 0.05$, respectively) (Fig. 6b, Supplementary Fig.4 and
225 Supplementary Table 3). This is consistent with *che-2* being a stronger allele than *che-*
226 1 in the *lhy* background¹⁰, explaining why there is an epistatic interaction with *cml23-2*
227 *cml24-4* and *che-2* but not with *che-1*.

228

229 Because the circadian oscillator contributes to the photoperiodic regulation of flowering
230 and *cml23-2 cml24-4* mutants are late-flowering³⁵, we tested epistasis between
231 *CML23/CML24* and *CHE* by measurement of flowering time. *che-2* is an early flowering
232 mutant and, as suggested above, possibly the stronger allele (Supplementary Fig. 5).
233 As in the circadian experiments, there is an epistatic relationship between mutations in
234 *CML23/CML24* and *CHE* in the regulation of flowering time (Fig. 6c and Supplementary
235 Fig. 5) when *che-2* was used. *che-2 cml23-2 cml24-4* mutant flowered at the same time
236 as *che-2*, and significantly earlier than *cml23-2 cml24-4* (One-way ANOVA, $p > 0.05$ and
237 $p < 0.05$, respectively) (Fig. 6c and Supplementary Fig. 5). However, and similarly to leaf
238 movement, flowering time provided no evidence of epistasis between *che-1* and *cml23-*
239 2 *cml24-4* (Fig. 6d and Supplementary Fig. 5).

240

241 The data suggest that the $[Ca^{2+}]_{cyt}$ -dependent regulation of circadian period by *CML24*
242 is not directly mediated by *CCA1*, *LHY* and *ZTL* and that *CML24* might regulate *TOC1*
243 and *CHE*, because functional copies of these two clock genes are required to express
244 the *cml23-2 cml24-4* phenotype.

245

246 Discussion

247 The Ca^{2+} -sensor *CML24* is a regulator of Arabidopsis circadian period

248 We tested the hypothesis that circadian oscillations of $[Ca^{2+}]_{cyt}$ can feed back into the
249 circadian oscillator. We demonstrate that $[Ca^{2+}]_{cyt}$ signals can regulate the expression

250 of the Ca^{2+} -binding *CALMODULIN-LIKE24* (*CML24*) and that *CML24* also regulates
251 circadian period, with the loss-of-function phenotype being absent when $[\text{Ca}^{2+}]_{\text{cyt}}$
252 rhythms are abolished. We conclude that correct circadian period is dependent on
253 *CML24* and circadian rhythms of $[\text{Ca}^{2+}]_{\text{cyt}}$. Epistatic analysis suggests that *TOC1* and
254 likely *CHE* genetically interact with *CML24* (Supplementary Fig. 6). Additionally, we
255 show that $[\text{Ca}^{2+}]_{\text{cyt}}$ signals can regulate the expression of *CHE* in a *CML24*-independent
256 manner and transcriptional regulation of *CHE* is unlikely to regulate circadian period.

257
258 It was previously reported that *CML24* regulates flowering time³⁵. Our new data
259 demonstrates that *CML24* also regulates circadian period in *Arabidopsis* because two
260 different alleles (*cml24-1* and *cml24-4*) alone or in combination with the null allele of
261 *CML23* (*cml23-2*)³⁵, had a long circadian period (Fig. 2). We found that *CML24* has
262 robust and profound effects on period (Fig. 3). The period lengthening persisted in
263 different clock mutant backgrounds such as *cca1-11*, *lhy-21* and *ztl-3* (Fig. 5). The
264 magnitude of the period lengthening of the *CML24* mutants (from 0.6 to 2 h) is larger or
265 similar to previously reported mutations in important circadian genes: *prr7-11* and *prr9-1* (0-2 h)³⁹, *che-1* and *che-2* (no effect on period)¹⁰, *prr3-1* and *prr5-3* (\square 1 h)⁴⁰, *Ink1-1*
267 (no effect on period), *Ink2-2* (1 h), *Ink1-1 Ink2-2* (2 h)⁴¹, *che-1/lhy* and *che-2/lhy* double
268 mutants have a significantly shorter circadian period (\sim 0.5 or 1 h, respectively)
269 compared to the *lhy* mutant¹⁰. *CML24-OX1* and *CML24-OX2* were without phenotype,
270 which might be expected for a sensor protein whose activity depends on and might be
271 limited by Ca^{2+} concentration, rather than abundance of the sensor protein. Meaning
272 that the presence of 24 h $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations might be critical for the production of the
273 physiological response as observed in Fig. 4a-d and that in the over-expressor lines,
274 the Ca^{2+} signature might be still decoded. Nevertheless, the limitation of other protein
275 targets of *CML24* and activators cannot be ruled out.

276

277 CML24 binds Ca^{2+} at EF hands to cause a conformational change but has no other
278 identified functional domains³⁵. Our data are consistent with CML23 and CML24 acting
279 as Ca^{2+} -sensors because we demonstrate that the effect of *CML23* and *CML24*
280 mutations on circadian period depends on $[\text{Ca}^{2+}]_{\text{cyt}}$ as shown by the absence of an
281 effect of the *cml23-2 cml24-4* mutation when circadian oscillations of $[\text{Ca}^{2+}]_{\text{cyt}}$ are
282 suppressed^{16, 24, 29} (Fig. 4a-4d). This also demonstrates that sucrose regulates
283 circadian period through a pathway independent of CML23 and CML24 because in low
284 light, added sugar shortened circadian period¹¹ which was unaffected by *cm23-2*
285 *cml24-4* (Fig. 4c). In the presence of nicotinamide, the circadian period in wild type and
286 *cml23-2 cml24-4* was the same. However, the period of the double mutant was
287 increased by nicotinamide (Fig. 4a), suggesting that nicotinamide might target both
288 $[\text{Ca}^{2+}]_{\text{cyt}}$ -dependent and -independent pathways, or additional Ca^{2+} -sensors might be
289 involved⁴². The Arabidopsis genome encodes over 50 CaM and CMLs^{42,43} and other
290 Ca^{2+} sensors, which could also contribute to circadian regulation.

291

292 ***CML24* genetically interacts with *TOC1* and possibly with *CHE* to regulate**
293 **circadian period**

294 The absence of the *cml23-2 cml24-4* circadian phenotype in *toc1-2 cml23-2 cml24-4*
295 indicates that the CMLs proteins are unable to exert their regulator function if TOC1 is
296 absent (Fig. 5a and Supplementary Fig. 4). *CML24* and *TOC1* are expressed in diverse
297 tissues and organs⁴⁴ which is consistent with our genetic studies, but more studies are
298 necessary to conclude how cytosolic CML24 regulates TOC1 function. *CHE* might have
299 a role because there was a genetic interaction between *CML23/CML24* and *CHE* in the
300 regulation of circadian period when the *che-2* allele was used (Fig. 6).

301

302 Because we found that Ca^{2+} alone or in combination with W7 was able to suppress
303 *CHE* transcript abundance in Col-0 plants (Fig. 1) and in the *cml* double mutant
304 (Supplementary Fig. 3), we suggest that the genetic interaction between

305 *CML23/CML24* and *CHE* is not dependent on transcriptional regulation and that the
306 effect of W7 is not through an effect on *CML23/24*. Additionally, circadian period was
307 not affected by a transient increase in $[Ca^{2+}]_{cyt}$ following W7 treatment. This is not
308 surprising, because *che* mutants, in which *CHE* transcript abundance is constitutively
309 reduced, period is unaffected¹⁰.

310

311 Whilst we do not consider that the Ca^{2+} -induced transcriptional changes in *CHE* affect
312 circadian period, it might be of functional significance because it is consistent with the
313 *CHE* binding site, also known as Site IIb⁴⁵, being similar to the $[Ca^{2+}]_{cyt}$ -regulated Site II
314 promoter element (AGGCCCAT)³². $[Ca^{2+}]_{cyt}$ -regulation of Site II is most likely through
315 the TCP family of transcription factors³², of which *CHE* is a member. *CHE* binds to the
316 class I TCP-binding site (TBS) (GGTCCCAC) in the *CCA1* promoter and represses its
317 expression¹⁰. In addition to *CHE*, *CCA1* transcript was the only clock gene that was
318 modestly activated around 8 h after the last pulse of $CaCl_2$ and *CHE* repression (Fig.
319 1b). *CHE* oscillates 9 h out of phase with *CCA1* transcript¹⁰ and at a similar phase with
320 $[Ca^{2+}]_{cyt}$ oscillations. Whilst we conclude that transcriptional changes in *CHE* are
321 unlikely to mediate changes in circadian period, it raises the possibility that *CHE*
322 transmits information about Ca^{2+} signals to the *CCA1* promoter.

323

324 Our data identify roles for Ca^{2+} and *CML24* in the circadian clock. These findings unveil
325 for first time in plants a function for circadian oscillations of $[Ca^{2+}]_{cyt}$ and expand the
326 architecture of the plant circadian oscillator.

327

328 **Methods**

329 **Plant material and growth**

330 *Arabidopsis* mutant lines used in the reverse genetic screen were supplied generously
331 from laboratories working in the area of Ca^{2+} signalling in plants and are listed in

Supplementary Table 2. The T-DNA insertion mutants *che-1* and *che-2*¹⁰ and the point mutation single mutants *toc1-2*⁹ and *lux-4*⁴⁶ were provided by Steve Kay (The Scripps Research Institute, USA); the T-DNA insertion mutants *cca1-11*, *lhy-21*, *elf3-4* and *elf4-1*⁴⁸ were donated by Seth Davis (University of York, UK); the T-DNA single mutant *ztl-3* (SALK_035701)¹⁷ was obtained from Nottingham Arabidopsis Seed Centre (NASC), UK. To obtain the triple mutants of the circadian oscillator genes with the *cml23-2* *cml24-4* double mutant (Col-0), the single mutants *che-1* (Col-0), *che-2* (Col-0), *cca1-11* (WS), *lhy-21* (WS), *toc1-2* (C24), *ztl-3* (Col-0), *elf3-4* (WS), *elf4-1* (WS) and *lux-4* (C24), were crossed independently to *cml23-2* *cml24-4* (Col-0) double mutants to generate triple mutants. The F2 progeny was then self-fertilized to obtain an F3 generation. The F3 and F4 generations were then genotyped to ensure all the mutant alleles were homozygous. F4 or subsequent generations were used for the epistatic study. Similarly, to the circadian clock mutants, different Arabidopsis ecotypes (WS and C24) were also crossed to Col-0.

Growth of *Arabidopsis thaliana*, photon-counting imaging of aequorin and luciferase luminescence and transformation techniques were as described in ²⁹ unless otherwise stated.

349

350 **[Ca²⁺]_{cyt} manipulation**

To obtain plants with undetectable circadian [Ca²⁺]_{cyt} rhythms (unentrained), meaning that [Ca²⁺]_{cyt} remained at basal levels, 35S:AEQ WS seeds²⁹ were grown in opaque 7 mm x 9 mm plastic rings sealed at the base with 0.5 µm nylon mesh (Normesh, UK) on sucrose-free 0.5 MS agar seeds, germinated without stratification and grown in continuous white light (LL) for at least 12 days. Artificial [Ca²⁺]_{cyt} rhythms were induced in these plants by step-wise addition of external CaCl₂ during the subjective day, followed by removal (Supplementary Table 1). During treatment, seedlings were floating on the mesh rafts on temperature-adjusted solutions. All experiments were repeated at least twice.

360 Induction of a single 24 h $[Ca^{2+}]_{cyt}$ peak was carried essentially the same except using
361 a FLUOstar plate reader (BMG Labtech, Germany). 200 μ l of treatment solution to
362 provide final concentrations of 0 mM to 150 mM $CaCl_2$ (Supplementary Table 1) was
363 injected into wells of a 96 well plate containing individual 12 day old 35S:AEQ
364 transformed seedlings that had previously been reconstituted with 20 μ M
365 coelenterazine (20 $^{\circ}$ C, overnight). Seedlings were washed with temperature-adjusted
366 deionized water before measurement and luminescence was measured⁴⁸. Solutions were
367 replaced with the successive treatment every hour. Results were assessed from three
368 independent experiments each consisting of a minimum of 12 replicates per treatment.
369 For $[Ca^{2+}]_{cyt}$ measurements after treatment with N-(6-Aminohexyl)-5-chloro-1-
370 naphthalenesulfonamide hydrochloride (W7) (Calbiochem) see Supplementary
371 Methods.

372

373 **Microarray analysis**

374 Plants treated with $CaCl_2$ as described above to generate 24 h $[Ca^{2+}]_{cyt}$ oscillations
375 were harvested every 4 h for 24 h. RNA was isolated as described in ¹⁶. RNA was
376 hybridised to GeneChips *Arabidopsis* ATH1 Genome Array using NASC's International
377 Affymetrix service. Raw intensity data were normalized across chips using RMA
378 (<http://www.bioconductor.org>) automatically log transforming the expression data. Array
379 1-22_Calcium_12h_Rep2_ATH1 was removed from the analysis after failing
380 hybridization quality control.

381

382 **qPCR analysis**

383 Experiment to determine the effect of W7: Col-0 and *cm123-2 cm124-4* plants were
384 treated with W7 solution (660 μ M W7 and 50 mM $CaCl_2$ containing a final
385 concentration of 2.5 % (v/v) DMSO) or deionized water at ZT36 and ZT48 in constant
386 light for 2 h and then frozen in liquid N_2 .

Experiment to determine the effect of *cml23-2 cml24-4* mutation on the circadian clock transcript abundance: Col-0 and *cml23-2 cml24-4* mutant were grown at 20 °C under 12 light /12 dark and then transferred into constant light conditions. After 2 days under constant light, plants were collected every 2 h from ZT48 to ZT72. Total RNA was extracted of three biological replicates of at least four pooled plants each, using the RNeasy Plant Mini Kit (QIAGEN) and RNase-Free DNase on-column treatment (QIAGEN). cDNA was synthesized from 500 ng RNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) using oligo(dT) primers. The gene-specific products were amplified using the Rotor-Gene SYBR Green PCR Kit on a Rotor-Gene 6000 Real-Time PCR machine (QIAGEN). Primers used are detailed in Supplementary Methods. Relative transcript levels were determined by incorporating PCR efficiencies⁴⁹.

Leaf movement analysis

Analysis of circadian rhythms of the first true leaves was performed as described in¹⁶ without experimenter knowledge of seed lines. In those experiments where nicotinamide was applied, 50 mM Nicotinamide (Sigma) or deionized water was applied once a day for 2 days before the start of imaging; 50 µl of solution was applied to the aerial parts of each seedling. The experiments were repeated twice but for the triple mutants, *cml23-2 cml24-4 che-2* and *cml23-2 cml24-4 toc1-2* were repeated three times. For *cml23-2 cml24-4 toc1-2* two independent lines were used.

Photoperiodic flowering time screening

Plants were sown directly onto soil and grown in 20 °C and 100 µmol m⁻² s⁻¹ in either long day (LD) conditions (16 h L:8 h D) or short day (SD) conditions (8 h L: 16 h D). Flowering time was defined as when the emerging bolt reached a height of approximately 5 mm. Screening experiments typically had 6 to 8 plants of each line per

414 growth condition, and confirmation experiments had 15 to 16 plants of each line per
415 growth condition.

416

417 **Statistical Analysis**

418 All statistical tests, n number, the measure of the centre and the error bars are
419 described in figure legends when appropriate. Other statistical parameters are listed in
420 Supplementary Statistical Parameters section. For comparison between two groups,
421 two-tailed Student's *t*-test or two-sided Mann-Whitney Rank Sum Test were used. Both
422 test analyses were considered significant if $p < 0.05$, $p < 0.01$ and $p < 0.001$. For
423 comparison between more than two groups, one-way ANOVA followed by Holm-Sidak
424 method or Kruskal-Wallis one-way ANOVA followed by Dunn's method or Tukey test
425 were used. ANOVA tests were performed with an alpha level of 0.05 or 0.001.

426

427 **Data availability**

428 The authors declare that the data supporting the findings of this study are available
429 within the paper (Figures 1 to 6) and its supplementary files (Supplementary Figure 1 to
430 5 and Supplementary Tables 2 and 3). Additionally, microarray data are available at
431 NASC Arrays (<http://arabidopsis.info/affy>), experiment reference NASCARRAYS-529.

432

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559

560 **Figure legends**

561 **Fig. 1.** Transcripts Abundance of Circadian Clock Genes is Modulated by $[\text{Ca}^{2+}]_{\text{cyt}}$.

562 **a**, Imposing oscillations of external CaCl_2 restores circadian oscillations in $[\text{Ca}^{2+}]_{\text{cyt}}$ in
 563 unentrained seedlings. See also Supplementary Fig. 1c for calibrated data. Results
 564 represent the mean \pm S.D. (n=12 biological replicates) for one experiment. Experiments
 565 were repeated three times.

566 **b**, Effect of the imposition of a 24 h oscillation of $[\text{Ca}^{2+}]_{\text{cyt}}$ on the transcript abundance
 567 (expressed as log2) of the circadian clock genes. Closed circles indicate unentrained
 568 water-treated samples, green circles the unentrained CaCl_2 -treated plants. To generate
 569 the oscillation, CaCl_2 was applied as described by the shaded areas and in
 570 Supplementary Table 1. Results represent the mean (n=2 biological replicates).

571 **c**, Plants treated at ZT36 and ZT48 with a solution containing 660 μM W7 and 50 mM
 572 CaCl_2 for 2 h, were assayed for changes in the abundance of circadian clock
 573 transcripts by qPCR. Dots represent each measurement and the black bars the mean \pm
 574 S.D. (n= 3 biological replicates). See also Supplementary Fig. 1. Single, double or triple

575 asterisk indicate significance of ≤ 0.05 , ≤ 0.01 and ≤ 0.001 , respectively after two-tailed
576 Student's t test analysis or two-sided Mann-Whitney Rank Sum Test (ZT36 *CCA1*,
577 *PRR7* and *ELF4*; ZT48 *ELF4* and *LHY*).

578 **Fig. 2.** CML24 Regulates Circadian Period in Arabidopsis.

579 Average normalized traces of leaf positions (left panels). FFT-NLLS analysis of the
580 circadian period for leaf movement experiments: dots indicate individual samples and
581 black bars mean period \pm S.E.M (right panels). **a**, Col-0 n=70, *cml23-2* n=70, *cml24-1*
582 n=97, double mutant n=94; **b**, Col-0 n=63, *cml23-2* n=69, *cml24-4* n=49, double mutant
583 n=110, rhythmic leaves. **a** shows the results of *cml23-2* and *cml24-1* single and double
584 mutants, **b** shows the results of *cml23-2* and *cml24-4* single and double mutants. Red
585 lines indicate Col-0, grey lines indicate *cml23cml24* double mutants, and light blue
586 *cml24* single mutants. *cml23-2* traces for leaf position were removed for clarity. All
587 plants were grown under 12 h L: 12 h D cycles before the experiments. Data represent
588 three (**a**) or two (**b**) independent experiments. Single or triple asterisk indicate
589 significance of ≤ 0.05 and ≤ 0.001 , respectively, after two-tailed Student's t test or two-
590 sided Mann-Whitney Rank Sum test (**b**, Col-0 vs. double mutant).

591 **Fig. 3.** CML24 has profound effect on the regulation of the Arabidopsis circadian clock.

592 The *cml23-2 cml24-4* mutant has a long circadian period of 35S:AEQ and CCA1:LUC
593 luminescence. Mean normalized luminescence \pm S.D. of 35S:AEQ (**a**) and CCA1:LUC
594 (**b**) for wildtype (red circles) and *cml23-2 cml24-4* (grey circles) from two independent
595 experiments (**a**, n=8 biological replicates; **b**, Col-0 n=23 and *cml23-2 cml24-4* n=24,
596 biological replicates). **c** and **d** show the FFT-NLLS analysis of the samples used in **a**
597 and **b**, respectively. Triple asterisk indicate significance of ≤ 0.001 , after two-tailed
598 Student's t test analysis (**c**) and two-sided Mann-Whitney Rank Sum Test (**d**). **e**,
599 *CCA1*, *TOC1* and *PRR7* transcripts abundance were analysed at the time point
600 indicated in plants grown for 12 days in 12h:12h light:dark cycles and transferred to

601 continuous light at ZT0. qPCR results represent the mean \pm S.D. (n=3 biological
602 replicates). Single, double or triple asterisk indicate significance of ≤ 0.05 , ≤ 0.01 and \leq
603 0.001, respectively after two-tailed Student's t test analysis or Mann-Whitney Rank
604 Sum Test (*CCA1* ZT60 and *PRR7* ZT48).

605 **Fig. 4.** Circadian oscillations of $[Ca^{2+}]_{cyt}$ are necessary for the correct function of the
606 Circadian Oscillator.

607 **a**, Circadian period estimates of leaf movement in continuous high light ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$) of Col-0 and *cml23-2 cml24-4* plants treated with either 50 mM nicotinamide (Col-0
608 n=15, *cml23-2 cml24-4* n=33, biological replicates) or water (Col-0 n=16, *cml23-2*
609 *cml24-4* n=29, biological replicates). **b**, Circadian period estimates of CCA1:LUC
610 rhythms in continuous high light ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$) or **c**, continuous low light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) of Col-0 and *cml23-2 cml24-4* plants grown in the presence of either water (high
611 light Col-0 n=7, *cml23-2 cml24-4* n=8, low light n=16, biological replicates), 90 mM
612 sucrose (high light n=8, low light n=16, biological replicates) or 90 mM mannitol (Col-0
613 n=8, *cml23-2 cml24-4* n=7, biological replicates). **d**, Circadian period estimates of
614 CCA1:LUC rhythms in Col-0 and *cml23-2 cml24-4* plants under continuous high mixed
615 red (660 nm) and blue (470 nm) light ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$) (n=4, biological replicates) and
616 continuous monochromatic blue or red light ($40 \mu\text{mol m}^{-2} \text{s}^{-1}$) (blue n=7, red Col-0 n=11
617 and *cml23-2 cml24-4* n=10, biological replicates). **e**, Effect of the imposition of a ramp
618 of external CaCl_2 (Fig. 1a) on the expression (log2) of *CML24* and *CML23*. CaCl_2 was
619 applied as described by the shaded areas and in Supplementary Fig. 1c. Plant material
620 was harvested from the onset of treatment every 4 h for 24 h to extract RNA for probing
621 with microarray. Results represent the mean (n=2 biological replicates). **f**, Circadian
622 period estimates of CCA1:LUC rhythms in continuous high light ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$) of
623 Col-0 (n=7 biological replicates) and *cml23-2 cml24-4* (n=8 biological replicates) plants
624 treated from the day before going into continuous high light either with water or 200 μM
625 cPTIO. Period estimates were obtained by BRASS and are shown as mean \pm S.E.M.

628 Data were obtained from 1 independent experiment. Experiments were repeated at
629 least twice. Single, double or triple asterisk indicate significance of ≤ 0.05 , ≤ 0.01 and \leq
630 0.001, respectively after two-tailed Student's t test analysis or two-sided Mann-Whitney
631 Rank Sum test (**a** (water), **b** (mannitol) and **f**).

632 **Fig. 5.** Epistatic Analysis of Leaf Movements Rhythms Shows that *TOC1* is
633 Functionally Linked to *CML24* to Regulate Circadian Period.

634 Average normalized traces of leaf positions and FFT-NLLS analysis of the circadian
635 period for leaf movement experiments. **a** shows the results of *cml23-2 cml24-4* with
636 *toc1-2* (Col-0 n=33, C24=31, double mutant=22, clock gene mutant=24, triple
637 mutant=25), **b** with *cca1-11* (Col-0 n=7, Ws-2=6, double mutant=29, clock gene
638 mutant=21, triple mutant=19), **c** with *lhy-21* (Col-0 n=24, Ws-2=24, double mutant=22,
639 clock gene mutant=24, triple mutant=14) and **d** with *ztl-3* (Col-0 n=22, double
640 mutant=25, clock gene mutant=26, triple mutant=15). Grey lines indicate *cml23-2*
641 *cml24-4*, blue clock gene single mutants and black the triple mutants, respectively.
642 Wild-type traces for leaf position were removed for clarity. All plants were grown under
643 12 h L: 12 h D cycles before the experiments. Data are presented from one experiment
644 representative of two (*cca1-11*, *lhy-21*, *ztl-3*) or three (*toc1-2*) independent
645 experiments. See also Supplementary Fig. 4 and Supplementary Table 3. Single or
646 triple asterisk indicate significance of ≤ 0.05 and ≤ 0.001 , respectively, after Kruskal-
647 Wallis One Way Analysis of Variance on Ranks followed by Dunn's method was used
648 to compare the triple mutant to the single and *cml23-2 cml24-4* double mutant.

649 **Fig. 6.** Epistatic Analyses of Leaf Movements Rhythms and Flowering Time Shows that
650 *CHE* is Functionally Linked to *CML24*.

651 Average normalized traces of leaf positions and FFT-NLLS analysis of the circadian
652 period for leaf movement experiments. **a** shows the results of *cml23-2 cml24-4* with
653 *che-2* (Col-0 n=29, *cml23-2 cml24-4*=48, *che-2*=47, triple mutant=40) and **b** with *che-1*

(Col-0 n=29, *cml23-2 cml24-4*=48, *che-1* =39, triple mutant=46). Wild-type traces for leaf position were removed for clarity. Data are presented from one experiment representative of two (*che-1*) or three (*che-2*) independent experiments (Supplementary Fig. 4 and Supplementary Table 3). Flowering time responses under long (16 h:8 h) or short days conditions (8 h:16 h) for *che-2 cml23-2 cml24-4* (**c**) and *che-1 cml23-2 cml24-4* (**d**) mutants. Number of leaves were recorded when the emerging bolt was 5 mm high. Dots represent the individual plants and the black bars the mean \pm S.D. (n=16; in LD Col-0 n=15; in SD *cml23-2 cml24-4* n=15 and *che-2* triple mutant n=13). Single or triple asterisk indicate significance of ≤ 0.05 or ≤ 0.001 , respectively after Kruskal-Wallis One-Way ANOVA analysis followed by Tukey test (LD) or Dunn's method (**a**, **b** and SD), when the triple mutant was compared to the *che* and *cml23-2 cml24-4* mutants. Flowering rate was calculated using the number of days since germination when the number of leaves was recorded. *che-2 cml23-2 cml24-4* mutant used was HL18 and *che-1 cml23-2 cml24-4* was HL15. An independent experiment in LD was done using three different mutants lines (Supplementary Fig. 5).

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697 **Competing interests**

698 The authors declare no competing financial interests.

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